REMARKS/ARGUMENTS

The Pending Claims

Claims 8-12 are pending and are directed to a method of producing a rat embryonic stem (ES) cell.

Summary of the Office Action

The Office rejects claims 8-10, 12, and 13 under 35 U.S.C. § 103(a) as allegedly obvious over Loring (WO 99/27076) in view of Price et al. (WO 98/30679) and Takahama et al. (*Oncogene, 16*: 3189-3196 (1998)).

The Office rejects claim 11 under 35 U.S.C. § 103(a) as allegedly obvious over Loring in view of Price et al., Takahama et al., Vassilieva et al. (*Experimental Cell Research, 258*: 316-373 (2000)), and Mandalam et al. (U.S. Patent 7,297,539).

Reconsideration of these rejections is hereby requested.

Discussion of the Obviousness Rejections

The Office maintains its contention that the subject matter of the pending claims is obvious over Loring in view of one or more of Takahama et al., Price et al., Vassilieva et al., and Mandalam et al. These rejections are traversed for the following reasons.

The pending claims are directed to a method of producing a rat embryonic stem cell consisting essentially of steps (A)-(E), which are performed using a culture medium with 2% or less serum concentration: (A) culturing a rat blastocyst in a leukemia inhibitory factor (LIF)-free culture medium to form an inner cell mass in the blastocyst, (B) dissociating the inner cell mass, wherein the dissociated inner cell mass is in a cell aggregate state, (C) culturing primary embryonic stem cells resulting from a culture of the dissociated inner cell mass until the primary embryonic stem cells can be passaged, (D) dissociating the primary embryonic stem cells, which can be passaged, wherein the dissociated primary embryonic stem cells are in a cell aggregate state, and (E) culturing the dissociated primary embryonic stem cells to establish an embryonic stem cell, wherein a rat leukemia inhibitory factor (rLIF)-containing culture medium is used in steps (C)-(E).

Applicants note that prior to the claimed invention the establishment of rat ES cells had not been reported even though various other mammalian ES cells had been successfully established following the first report in 1981 of mouse ES cell establishment (see, e.g., page 3, lines 20-24, of the specification). The inventors determined suitable culture and passage conditions and succeeded in establishing rat ES cells capable of forming a chimeric rat. Since the development of rat ES cells is essential for the production of a knockout rat, the claimed invention provides the first means for producing various experimental rat models. In particular, the inventors determined that rat ES cells having the ability to produce a chimeric rat could be established using the inventive method. In particular, the inventors determined that (i) using culture media with 2% or less serum concentration, (ii) using LIF-free medium for blastocyst culture and LIF-containing medium for culture after inner cell mass (ICM) dissociation, and (iii) dissociating ICM in a cell aggregate state enables the successful establishment of rat ES cells.

The Office contends that the Loring reference teaches the use of an LIF-free culture medium for culturing primary blastocysts to induce ICMs. The Office contends that the rat cells established in the Loring reference are ES cells. As disclosed in the specification, to be a rat ES cell, a cell must have several characteristics: (a) expresses Oct3/4 gene and Nanog gene, (b) is positive for alkaline phosphatase (AP) activity, (c) has an embryoid body forming ability, (d) expresses SSEA (Stage-Specific Embryonic Antigen)-1 and SSEA-4, (e) has the same number of chromosomes as does a normal rat cell, (f) is capable of being subcultured and holding the undifferentiated state, (g) has *in vitro* pluripotency, (h) has a potential to differentiate into cells of three embryonic germ lineages, (i) has teratoma formation ability, and (j) has an ability to produce a chimeric rat (see, e.g., page 4, line 22, through page 5, line 2). However, the Loring reference only provides evidence that the rat cells derived from ICMs by co-culture with mouse ES cells are AP-positive (see Example 3) and can form embryoid bodies (see Example 4). The experiments in Loring regarding teratoma formation (see Example 4(B)(2)) and chimeric rat production (see Example 6) are prophetic examples, such that one of ordinary skill in the art would not know whether the rat pluripotent cells of the Loring reference can produce a chimeric rat.

Generally, ES cells refer to pluripotent cells with the ability to produce a chimeric animal. Since Loring does not provide experimental evidence that the pluripotent cells

described therein produce a chimeric rat, one of ordinary skill in the art would not know whether Loring established true rat ES cells. As evidenced by Brennin et al., *Developmental Biology, 185*: 124-125 (1997) (submitted herewith), pluripotent cells with only a few of above-described characteristics, such as SSEA-1- and AP-positive cells, failed to generate a chimeric rat. Brennin et al. concludes that "a pluripotent population of ES cells from the rat has not been isolated" (see page 125, lines 10-11).

In an effort to demonstrate that the rat pluripotent cells established in the Loring reference similarly are not true rat ES cells, Applicants have reproduced the experiments set forth in the Loring reference as described in the "Declaration Under 37 CFR 1.132" of Dr. Takahiro Ochiya (submitted herewith). As evidenced in Figure 3 thereof, no ES cell colonies were produced, and, thus, no chimeric rat could be produced using the methods of the Loring reference.

Applicants note that the Loring reference teaches the addition of 15 to 20% serum to the culture media and treating the cells with trypsin (0.25% trypsin in 1 mM EDTA, 3 °C, 15 minutes). These treatments are considered harmful for the maintenance of an undifferentiated state and/or pluripotency. Interestingly, in Example 4(B) of the Loring reference, no embryoid bodies were formed from the rat cells before co-culture with mouse ES cells, whereas many embryoid bodies were obtained following co-culture with mouse ES cells. As a result, Applicants suspect that the pluripotent cells obtained by co-culture with mouse ES cells are not rat ES cells but, rather, the remaining mouse ES cells that survived through HAT selection, as is the situation observed in Brennin et al. (see page 124, lines 8-10).

Furthermore, Applicants note that, in a review article from Dr. Loring (Loring, *Methods in Molecular Medicine, 32*: 249-270 (1999) that published on December 29, 1999, Dr. Loring acknowledges that no rat ES cell line had been established at the time the review article was published, which is after the filing and subsequent publication of the Loring reference cited in support of the obviousness rejection (i.e., WO 99/27076, which published on June 3, 1999). In particular, Dr. Loring states that "the barrier to making the rat as genetically malleable as mouse has been the lack of rat ES cell lines. Although deriving rat

ES cell lines has proved difficult ... efforts are underway in several laboratories and are likely to be successful in the next few years" (see page 267, lines 12-16).

For the above-described reasons, the Loring reference cannot be considered to teach or suggest a method of establishing a true rat ES cell line (i.e., a rat ES cell line that can produce a chimeric rat).

Moreover, in contrast to the Office's contention, the Loring reference does not teach the use of an LIF-free culture medium for culturing primary blastocysts to induce ICMs. In particular, the Loring reference discloses that LIF-free medium fails to induce AP-positive cells without feeder cells supplying LIF and SCF (see Example 2 and Table 1). The rat cells derived from blastocysts cultured on rat fibroblasts in control medium (high glucose DMEM supplemented with 15% FBS, 1x non-essential amino acids, and 0.1 mM 2-mercaptoethanol) with or without 20 ng/mL bFGF were little stained (-/+) in an AP-staining assay, whereas those cultured on SNL76/7 cells, which produce LIF and SCF, were strongly stained in control medium with or without 20 ng/mL bFGF (++ or +++, respectively). These results strongly suggest to one of ordinary skill in the art the addition of LIF to a culture medium for inducing ICM in rat blastocysts. Therefore, one of ordinary skill in the art would have had no reason to remove the LIF from the culture medium for inducing ICM in order to produce a rat ES cell line.

The remaining cited references do not remedy the deficiencies of the Loring reference.

Price et al. discloses a supplement for serum-free media that supports the growth of ES cells. Applicants note that in Price et al., only the rat ES cell lines of Iannaccone et al., *Developmental Biology, 163*: 288-292 (1994), are exemplified as rat ES cells. However, as discussed above in Brennin et al., these cell lines were later determined not to be rat ES cell lines but, rather, mouse ES cells that accidentally contaminated the rat cell cultures.

Takahama et al. discloses that cloned rat LIF has the ability to maintain the stem cell-like phenotype of rat *ES-like* cells derived from rat ICM. Takahama et al. does not produce rat ES cell lines. Furthermore, Takahama et al. does not teach or suggest the use of LIF-free medium when inducing ICM formation in blastocysts, as required by the pending claims.

The Vassilieva and Mandalam references merely disclose mechanical dissociation of primary ES cells. These references do not disclose the use of an LIF-free culture medium for culturing primary blastocysts to induce ICMs, as required by the pending claims.

Since the cited references, when considered alone or in combination, do not teach or suggest all of the features of the claimed methods, the cited references do not render obvious the claimed invention.

Even if one of ordinary skill in the art were to interpret the Loring reference as disclosing an LIF-free medium, Applicants note that the claimed invention still would not be obvious in view of the cited references. As discussed above, the Loring reference fails to disclose a true rat ES cell (having the ability to produce a chimeric rat). Since no rat ES cell line was established (even using a LIF-containing medium), one of ordinary skill in the art would not have been able to reasonably predict the use of a LIF-free medium for culturing rat blastocysts to produce a rat ES cell line (having the ability to produce a chimeric rat) even with the teaching of Takahama et al. that rat LIF supports the maintenance of a stem cell-like phenotype of rat ES-like cells.

Regarding JP 05-304951 and the Yamanaka reference to which the Office cites to indicate the state of the prior art, Applicants provide the following comments.

In JP 05-304951, a LIF-containing medium always is used for establishing ES cell-like colonies. The effects of growth factors on the maintenance and proliferation of ES-like cells are compared between (i) LIF alone and (ii) LIF and IGF-II. With (ii) LIF and IGF-II, the cells maintained an ES cell phenotype, whereas (i) LIF alone resulted in differentiation. Thus, in JP 05-304951, LIF also is contained in a culture medium from ICM formation in blastocysts to maintenance and proliferation of ES-like cells. JP 05-304951 does not teach or suggest that LIF is not necessary to induce ICM formation in rat blastocysts.

The Office contends that the Yamanaka et al. teaches that the presence of bLIF in culture medium has no effect on the cell number of ICM in cultured bovine embryos. Applicants note, however, that Yamanaka et al. does not teach or suggest that the removal of LIF from the culture medium would result in a beneficial effect, such as increased or otherwise improved ICM formation in blastocysts.

Therefore, one of ordinary skill in the art upon reading JP 05-304951 and the Yamanaka reference would not have had any reason to remove LIF from a culture medium, especially in view of the teachings in the Loring reference that LIF-containing medium is necessary for culturing primary blastocysts.

The Office further contends that the "Declaration Under 37 CFR 1.132" of Dr. Takahiro Ochiya submitted with the "Reply to Office Action" dated November 16, 2009 is irrelevant because the Loring reference already disclosed the use of LIF-free medium for rat blastocyst culture. As discussed above, while the Loring reference may disclose the use of the LIF-free control medium or STO cells that have no recombinant LIF genes, the Loring reference teaches away from the combined use thereof (i.e., a culture medium that has no LIF) because the rat cells derived from blastocysts cultured on rat fibroblasts in the control medium were little stained in an AP staining assay (see Table 1). One of ordinary skill in the art would understand that the Loring reference teaches using LIF-containing medium or LIF-overproducing feeder cells for rat blastocyst culture. Therefore, Applicants believe that the evidence of beneficial effects discussed in the previously submitted "Declaration Under 37 CFR 1.132" of Dr. Takahiro Ochiya further supports the nonobviousness of the claimed invention.

The Office contends that there is no evidence indicating that the presence of 2%, 1%, or 0.5% serum in a culture medium would have different effects from a culture medium having 3% serum (as described in the post-filing Ueda reference). In an effort to demonstrate that the claimed invention exerts its beneficial effects using a culture medium with 2% or less serum concentration, Applicants have replicated the method described in Example 1 of the specification except that culture medium containing 2% fetal bovine serum was used in place of serum-free medium (see Example 2 of the "Declaration Under 37 CFR 1.132" of Dr. Takahiro Ochiya (submitted herewith)). The rat ES cells established using culture medium containing 2% serum were AP-positive like the ES cells established using serum free medium (see Figure 5 of the "Declaration Under 37 CFR 1.132" of Dr. Takahiro Ochiya (submitted herewith)). Since the beneficial effects of the invention were demonstrated in both the lower limit (serum-free) and upper limit (2% serum) of serum concentration, the claimed invention clearly exerts its beneficial effects using a culture medium with 2% or less serum concentration, as recited in the pending claims.

Thus, for all of the above-described reasons, Applicants assert that the inventive methods are not obvious in view of the cited references, such that the obviousness rejections should be withdrawn.

Conclusion

Applicants respectfully submit that the patent application is in condition for allowance. If, in the opinion of the Examiner, a telephone conference would expedite the prosecution of the subject application, the Examiner is invited to call the undersigned attorney.

Respectfully submitted,

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